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Lymphocyte transformation test: History and current approaches

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ABSTRACT

Drug-induced hypersensitivity reactions encompass a variety of different clinical phenotypes ranging from harmless rashes to fatal reactions. They can be classified into allergic (i.e. drug allergy) and non-allergic reactions (i.e. non-allergic hypersensitivity). Drug allergies in turn can either be antibody (e.g. IgE) or T cell-mediated.

One of the diagnostic tools for the in vitro detection of drug allergy is the lymphocyte transformation test (LTT) which is based on the activation and expansion of the drug-specific memory T cells following co-incubation of the patient's peripheral mononuclear cells (PMBC) with the suspected drug in vitro. The read-out parameter in the classical LTT is T cell proliferation which can be measured as counts per minute following the addition of radiolabeled thymidine to the cell culture. However, in the course of time different modifications of the classical LTT with regard to the read-out parameters and methods have been proposed. Likewise, variations of the LTT platform itself have been described in the literature.

This review article describes the development of the classical LTT and its use in the context of drug allergy detection and summarizes the modifications which have been published over time.

1. Epidemiology of drug hypersensitivity

The exact incidence and prevalence of drug hypersensitivity reactions are difficult to determine. This is due to differences in study design, and inconsistent application of the definition and identification of hypersensitivity reactions (Thong and Tan, 2011). However, it is reported that about 7% of the population are affected (Demoly et al., 2014; Gomes and Kuyucu, 2017; Brockow et al., 2019).

2. Clinical spectrum of drug allergy

Drug hypersensitivity reactions can affect any organ but the skin is most frequently involved (Demoly et al., 2014; Brockow et al., 2019). Cutaneous drug-induced hypersensitivity reactions encompass a variety of different clinical phenotypes which can be differentiated with regard to the time to onset into immediate and non-immediate reactions. Immediate reactions occur nearly always within the first hour and include urticaria, angioedema and anaphylaxis. In contrast, non-immediate reaction occur mostly after 24 h and include various exanthema (e.g. maculopapular) and the very rare, severe and potentially fatal bullous skin reactions, like the Stevens-Johnson syndrome and toxic epidermal necrolysis (Demoly et al., 2014; Brockow et al., 2019; Bellón, 2019).

3. Hypersensitivity versus drug allergy

3.1. Classification of adverse drug reactions

Adverse drug reactions (ADRs) have traditionally been classified in type A (augmented) and type B (bizarre) reactions. Type-A reactions were considered to be caused by known *pharmacological* or *toxic* reactions, and, hence, being predictable. In contrast, type B reactions were supposed to be caused by *individual predisposing* factors in the patient, being essentially unpredictable (Rawlins, 1977; Rawlins, 1981; Demoly et al., 2014; Gomes and Kuyucu, 2017; Bellón, 2019). Type B reactions account for about 15% of all ADRs and most of these are hypersensitivity reactions (Demoly et al., 2014; Pichler and Hausmann, 2016; Gomes and

Abbreviations: ADR, Adverse drug reaction; APC, Antigen presenting cell; BrdU, Bromodeoxyuridine; cpm, Counts per minute; CSFE, Carboxyfluorescein succinimidyl ester; LTT, Lymphocyte transformation test; PBMC, Peripheral blood mononuclear cells; SI, Stimulation index.

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Kuyucu, 2017; Pichler, 2019). Against the background of the current state of knowledge further refinements of these classifications have been suggested (Pichler and Hausmann, 2016; Pichler, 2019).

Recently, a new classification of ADRs has been proposed categorizing these as on- or off-target reactions in view of recent findings related to the mechanism and phenotype of the ADRs (White et al., 2015; Phillips, 2016). The on-target reactions are mediated by the pharmacological mode of action of the drug itself, whereas the off-target reactions are mediated by immunological mechanisms or a non-immunological activation of off-target receptors (e.g. non-IgE-mediated mast cell activation). There is some congruency between type A and on target, and type B and off-target reactions. However, in contrast to the type B classification, off-target reactions are at least partly predictable (e.g. by genetic factors or drug - HLA interaction) and are also dose dependent.

With regard to the aforementioned hypersensitivity reactions, the nomenclature for allergy for global use divides these (with regard to the pathophysiology) into *allergic* reactions (i.e. drug allergy) and *non-allergic* reactions (i.e. non-allergic hypersensitivity, e.g. intolerance to acetylsalicylic acid) (Johansson et al., 2004). Drug allergies in turn are immunological reactions, which can either be antibody (e.g. IgE) or T cell-mediated (Johansson et al., 2004). However, this nomenclature is not consistently applied in literature, in particular the umbrella term drug hypersensitivity is often used for drug-allergies.

3.2. Classification drug allergy

Traditionally drug-allergic reactions have often been classified according to the classification by Coombs and Gell which dates back to the 1960s (Coombs and Gell, 1968). This classification is still in use and combines the clinical phenotype of the reaction and time to onset with the underlying pathophysiology. In clinical practice, many of the cutaneous drug-induced reactions are either type I reactions (IgE-mediated, immediate type) like anaphylaxis or type IV reactions (lymphocyte-mediated, delayed type), like maculopapular exanthems (type II and III not further expanded here) (Sachs and Merk, 2005; Brockow et al., 2019). Type IV reactions were proposed for further subclassifications (IVa-d) according to different cytokine secretion patterns and involved leukocytes (for brevity not further detailed) (Pichler, 2003). Notably, in all type IV reactions (different) T cells play a dominant role (Pichler and Tilch, 2004; Pichler, 2005; Porebski et al., 2011).

4. Relevance of confirming drug allergy

The confirmation but also the exclusion of a drug allergy is of significant relevance for the individual patient, the treating physician and the health care system. If the drug allergy is not clarified this may either result in a severe reaction on renewed exposure or an unnecessary avoidance of drugs and use of inadequate alternatives (Brockow et al., 2015). For instance, the use of alternative antibiotics in cases of suspected penicillin allergy is associated with higher costs, more side effects and the spread of antibiotic resistance (Macy and Contreras, 2014). This emphasizes the importance of a systematic and standardized diagnostic evaluation of an assumed drug allergic reaction. In fact, only 10% to 20% of the self-reported drug hypersensitivity reactions can actually be confirmed as drug allergic by diagnostic allergy tests (Gomes and Kuyucu, 2017). Therefore, guidelines clearly recommend the diagnostic work-up of an assumed drug allergic reaction with the aim to identify the culprit drug, the underlying pathophysiology, and to assess the individual risk of the patient for further reactions followed by respective advice (Brockow et al., 2015).

5. Diagnostic work-up of drug allergies

The diagnosis of drug allergies rests on five pillars: i) classification of the clinical phenotype, ii) medical history, iii) in vitro tests, iv) in vivo tests (=skin tests), and v) provocation tests (Brockow et al., 2015). In vitro methods bear – in contrast to e.g. provocation tests - the immanent advantage of not jepordazing the patient (apart from taking the blood).

Among in vitro tests, procedures that detect specific (IgE) *antibodies* (i.e. serologic tests) can be distinguished from *cellular-based* test systems. Cellular test systems can be divided into those that detect IgE-mediated immediate type reactions (e.g. CD63 expression on basophils; see article by Elst and colleagues also part of the "Special Issue on in vitro detection of drug allergy") and those that are mainly used for delayed-type reactions (e.g. lymphocyte transformation test (LTT)).

The present review article will focus on the classical lymphocyte transformation test (LTT) used in the diagnosis of drug allergy based on the detection of cell proliferation by means of radioactive 3H-Thymidine. An outlook towards the potential future perspectives of the LTT is highlighted in another review article by Fatangare and colleagues, which is also part of the special issue.

6. LTT –immunological basis

The immunological principles underlying antigen recognition are described elsewhere (Cho and Uetrecht, 2017; Meng et al., 2018). In few sentences and focusing on T cells, typically a naïve CD4+ or CD8+ T cell can recognize its specific antigen if this (typically a peptide) is presented by professional antigen presenting cells in context with MHC-II / I molecules (= embedded in the MHC-cleft) (signal 1). To induce a primary immune reaction of the naïve CD4+ or CD8+ T cell, costimulatory molecules have to be expressed on the professional antigen presenting cell and be recognized by the T cell (signal 2). After antigen-specific activation the T cell proliferates (development of clones) and - dependent on various factors (e.g. antigen concentration, cytokine environment representing signal 3) - the effector mechanisms may be skewed in a certain direction (e.g. Th1, Th2, Th17). Notably, after this priming of the T cell, also memory cells develop. If they encounter the specific antigen at a later point of time, a secondary immune response is set up. This secondary immune response needs less costimulatory activation signals and less time than the priming (Pichler, 2003; Kambayashi and Laufer, 2014; Cho and Uetrecht, 2017; Meng et al., 2018; Bellón, 2019; Pichler, 2019).

There are different *mechanistic* models referring to the underlying pathophysiology of how T cells recognize the drug or its metabolite which are described elsewhere (Cho and Uetrecht, 2017; Meng et al., 2018; Pichler, 2019). Very likely, in drug allergy there will not be one mechanism that exclusively matches all different conditions (different drugs, different genotypes of the individuals, different environmental factors, different phenotypes of the reaction). Rather, some of the proposed mechanisms will more likely play a role in certain drug-reaction constellations. In addition, some of the mechanisms are connected or complementary to each other (Cho and Uetrecht, 2017). In the LTT, peripheral blood mononuclear cells (PBMC) of the patient are co-incubated with the suspected drug. The PBMC contain antigen-presenting cells (monocytes, B cells), which can present the antigen (=drug) to the specifically sensitized T cells (memory T cells). These recognize the antigen leading to activation and expansion of the drug-specific T cells (Lanzavecchia, 1985; Nyfeler and Pichler, 1997; Pichler and Tilch, 2004; Porebski et al., 2011).

Even if different types of drug allergy (i.e. type I – IV) are mediated via different pathophysiological *distal* effector mechanisms associated with different clinical pictures, a common starting point is the development of drug specific memory T cells. Therefore, the activation of these cells appears as a promising starting point for a test procedure as with the LTT (Cornejo-Garcia et al., 2007; Luque et al., 2008). Along these lines, one could assume that the activation of these memory cells could serve as a target for an in vitro test in all four types (according to Coombs and Gell, see above) of drug allergies (Pichler and Tilch, 2004; Pichler, 2005). Although some authors see the applicability of the LTT above all for delayed-type reactions (type IV) in which the distal effector

phase is also mediated by T cells (Naisbitt et al., 2014; Schrijvers et al., 2015), its successful application in immediate type reactions (type I), i.e. reactions in which the distal effector phase is typically mediated by IgE antibodies (Brugnolo et al., 1999; Orasch et al., 1999; Hari et al., 2001; Neukomm et al., 2001; Pichler and Tilch, 2004; Luque et al., 2008; Rozieres et al., 2009) has been shown just as the involvement of T cells (Torres et al., 2006; Cornejo-Garcia et al., 2007) .

Since the principle of the LTT is based on the presence of memory T cells, the frequency of these cells in the blood is of importance. In this respect one study found a frequency of 1/172.000 *PBMC* (Kalish et al., 1994). Another study found that about 1:250 to 1:10.000 of *T cells* of a patient are reactive to the drug (Beeler et al., 2006).

Due to the fact that the LTT detects memory T cells, it will not give a positive result in any drug hypersensitivity reactions, which are not mediated by a specific immune response like reactions following mast cell activation via the MRGPRX2 receptor (McNeil et al., 2015).

7. History of the LTT

The use of the LTT for the in vitro detection of a sensitization dates back to the 1960s (Summer et al., 2016) (Sarkany, 1967; Simon et al., 1970). Its name very likely derived from the *transformation* of the activated T cells into blasts following antigen-specific (or initially mitogen-induced (Hirschhorn et al., 1963)) activation which was observed by microscopy (Summer et al., 2016). As early as in the 1970s the addition of a radio-labelled DNA-base (thymidine) to measure the proliferation by means of incorporated radioactivity was reported (Sarkany, 1967). In 1976, the International Union of Immunological Societies (IUIS) recommended measuring the incorporation of 3H-thymidine for the LTT (Clinical Immunology, 1976). Fig. 1 provides an overview of the technical procedure for the classical LTT. In view of the observation that the lymphocytes start to *proliferate* following drug-specific stimulation, the LTT is sometimes addressed as lymphocyte *proliferation* test, or less often as lymphocyte *stimulation* or *activation* test.

The classical LTT is based on the indirect detection of drug-specific memory T cells which circulate in the peripheral blood of sensitized patients. For this reason, the LTT procedure starts with the taking of the blood sample from an assumed sensitized patient. In a first step, the PBMC (peripheral blood mononuclear cells) are separated by density gradient centrifugation whereby other blood cells like erythrocytes, thrombocytes and granulocytes are removed. These PBMC are then cultured in special cell culture medium supplemented with heat inactivated autologous plasma or AB-serum followed by seeding a distinct cell number (e.g. 2×10^5 PBMC/well) in 96 well round bottom plates. Then, the suspected culprit drug is added to the medium and the cell culture is incubated for 5 days at 37 °C and 5% CO_2 in an incubator. Subsequently, 3H-thymidine is added to the cell culture and incubated for 10–16 h. During this period, the 3H-thymidine is incorporated in the DNA of the proliferating cells. Finally, the amount of 3H-thymidine in each cell culture sample is measured by detecting the radiation in counts per minute (cpm) (Pichler and Tilch, 2004).

However, cell proliferation of immune cells can be subject to substantial biological variability, inter-individually but even intra-individually implicating different 3H-thymidine uptake of the cells even in non-stimulated samples from the same individual taking at different points of time. Hence, it is difficult to define a simple threshold level in terms of an absolute cpm value that can be interpreted as a positive result for the confirmation of drug-specific lymphocyte reactivity. Therefore, in most cases a stimulation index (SI) is calculated which takes the biological variation into account. The SI value is calculated by dividing the cpm value of the drug-stimulated samples by the cpm value of the unstimulated sample, where no drug was added. This approach shall account for intra- and inter-individual variations of the background cell proliferation. Mostly, a SI value of ≥ 2 is considered as a positive result, whereas the SI value for beta-Lactams was set to ≥ 3 by some authors (Pichler and Tilch, 2004).

8. Sensitivity and specificity of the LTT used in drug allergy

The sensitivity and specificity of the classical LTT varies depending on the clinical phenotype and the drugs used. Summarizing various LTT publications, the sensitivity ranges between 58% to 89% for mild and moderate allergic reactions and 25% to 75% for severe bulluos reactions. The corresponding specificities are within 93% to 100% for mild and moderate allergic reactions and 63% to 100% for severe bulluos reactions (Mayorga et al., 2016, Mayorga et al., 2017; Porebski, 2017; Cabañas et al., 2018; Bellón et al., 2020; Mayorga et al., 2019). The overall sensitivity of the LTT in well-defined drug hypersensitivity reactions may lie between 60% and 70% (Duran-Figueroa et al., 2015). However, in the different publications the gold or reference method used to calculate the sensitivity and specificity of the LTT varies, including besides provocation tests, skin tests (indicating reactions mediated by the specific immune system (exceptions not detailed here)), stringent medical history or combinations of these. For this reason, the stated values implicate some uncertainty as the used reference methods also vary with regard to sensitivity and specificity. Additionally, inclusion of drug hypersensitivity reactions not mediated by specific immune reactions e.g. reactions following mast cell activation mediated by the MRGPRX2 receptor can negatively affect the sensitivity and specificity calculation of the LTT if the reference method (e.g. provocation test) is able to detect this kind of drug hypersensitivity reaction.

In summary, in its traditional form, the LTT has a limited sensitivity and, due to the methodological effort, is unsuitable for daily routine use.

9. Modifications of the classical LTT: current approaches

In the course of time, different modifications to the classical LTT have been proposed in order to increase the sensitivity, specificity and/or practicability. Fig. 2 provides an overview of these approaches. Notably, all approaches still recur on the in vitro co-incubation of the PBMC with the drug or its metabolite which we will refer to as the *LTT-platform* in the following. The drug-specific[1] activation of the T cells is thus the necessary pre-requisite in all approaches. To facilitate an overview, these approaches may be divided into

i) those referring to the LTT-platform (i.e. initiation of the drug-specific activation) and
ii) those referring to the read-out parameters including methods for their detection

i) *modification of the LTT-platform*

These modifications encompass a variety of proposals to improve the drug-specific activation, e.g.

1 addition of professional antigen presenting cells (Rodriguez-Pena et al., 2006; Lopez et al., 2009; Antunez et al., 2011; Mayorga et al., 2016; Doña et al., 2017), Toll-like-Receptor agonists (Sanchez-Quintero et al., 2013; Doña et al., 2017), IL-2 (Ikeda et al., 1998), CD3 or CD3/CD28 (Trautmann et al., 2014), IL-7/IL-15 (Porebski et al., 2013).
2 depletion of regulatory T cells (CD3+ CD25high) which may suppress the activation of T cells (Srinoulprasert and Pichler, 2014; Mayorga et al., 2016).
3 addition of the reactive metabolite itself instead of the culprit (inert) drug or the addition of liver microsomes to generate the reactive

[1] the term drug-specific used here refers to the meaning that only this drug, but not a different drug which is irrelevant in view of the patient's history induces a reaction and that this reaction is not seen if the drug is incubated with PBMC of a healthy control.

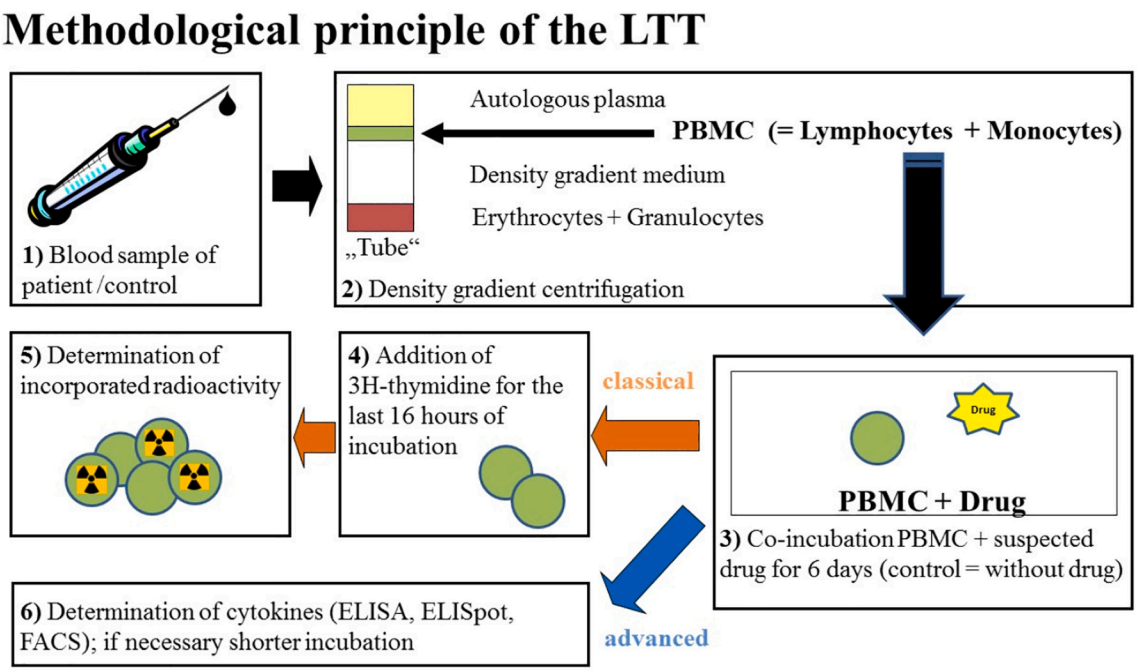

Fig. 1. Procedure of the classical LTT.

metabolite (Sachs et al., 2001; Yun et al., 2013; Mayorga et al., 2016; Cho and Uetrecht, 2017; Doña et al., 2017).

However, it appears that none of these modifications represent a major breakthrough since different approaches are still in use, and in many current publications none of the aforementioned modifications are applied.

ii) *modifications of the read-out parameters and methods*

The determination of the 3H-thymidine uptake was assumingly the most widely used read-out method for the LTT up to the year 2000 (Pichler and Tilch, 2004) and this read-out parameter is still in use (Bellón et al., 2020; Movsisyan et al., 2019; Jörg et al., 2020; Mori et al., 2020). However, one disadvantage of this method is the necessity to work with radioactive material (3H-thymidine), which complicates its application in daily clinical routine. In addition, apart from the confirmation of a drug-specific lymphocyte reactivity it does not characterise the reaction. Likewise, proliferation is given per culture well and not per individual cell and no additional assays can be performed with or after 3H-thymidine incorporation (Romar et al., 2016). Finally, the aim of improving the sensitivity and/or specificity of a method is often a driving force for further research. Looking back, these were possibly the reasons, why new read-out parameters and methods were sought by researchers. These new approaches can be differentiated with regard to the final read out parameter and the method applied to detect it.

Selected relevant modifications concerning the read-out parameters and methods are addressed in detail in the other articles of this Special issue. Briefly, the new read-out parameters may be grouped into three different categories:

a) (early) markers of lymphocyte activation[2], e.g. CD 69, CD25[3], CD134[3]
b) alternative markers of lymphocyte proliferation (BrdU, CSFE)[2]
c) Cytokines. Their application goes beyond the simple detection of drug-specific lymphocyte reactivity, because they also allow the characterization of the **in vitro** type of the reaction (Sachs et al., 2002; Lochmatter et al., 2009). The analysis of cytotoxicity, e.g. by analysis of respective molecules like granzyme B and granulysin, also helps to characterise the reaction.[2]

A comprehensive overview of the different biomarkers employed can be found in the review by Duran-Figueroa (Duran-Figueroa et al., 2015). The methods applied to detect these read-out parameters encompass among others the ELISA, the ELISpot (which additionally allows for the quantification of drug-specific activated T cells), flow cytometry or gene expression analysis by means of quantitative reverse transcription and polymerase chain reaction (Gaspard et al., 2000).

With regard to the sensitivity and specificity of recently applied read out parameters and methods we refer to the dedicated articles in this Special Issue. However, judging from recent publications it appears that detection of cytokine secretion (by ELISpot, ELISA or flow cytometry) is increasingly used.

10. Advantages and disadvantages of the LTT and modifications thereof

The advantages of the LTT include that – as an in vitro method – it is safe for the patient (no re-exposure to the potential allergen (drug)), apart from the taking of the blood. Consequently, there is no risk to trigger a harmful allergic reaction during allergy testing or to induce a potential sensitization to the tested drug in the patient. A further

[2] If combined with other methods (e.g. staining of proliferating cells in the flow cytometry) they may also provide further information about the in vitro reaction, thus going beyond the determination of drug-specific lymphocyte reactivity.

[3] Common T cell activation markers which to date have not been reported as a read-out parameter for the LTT in the context of drug allergy.

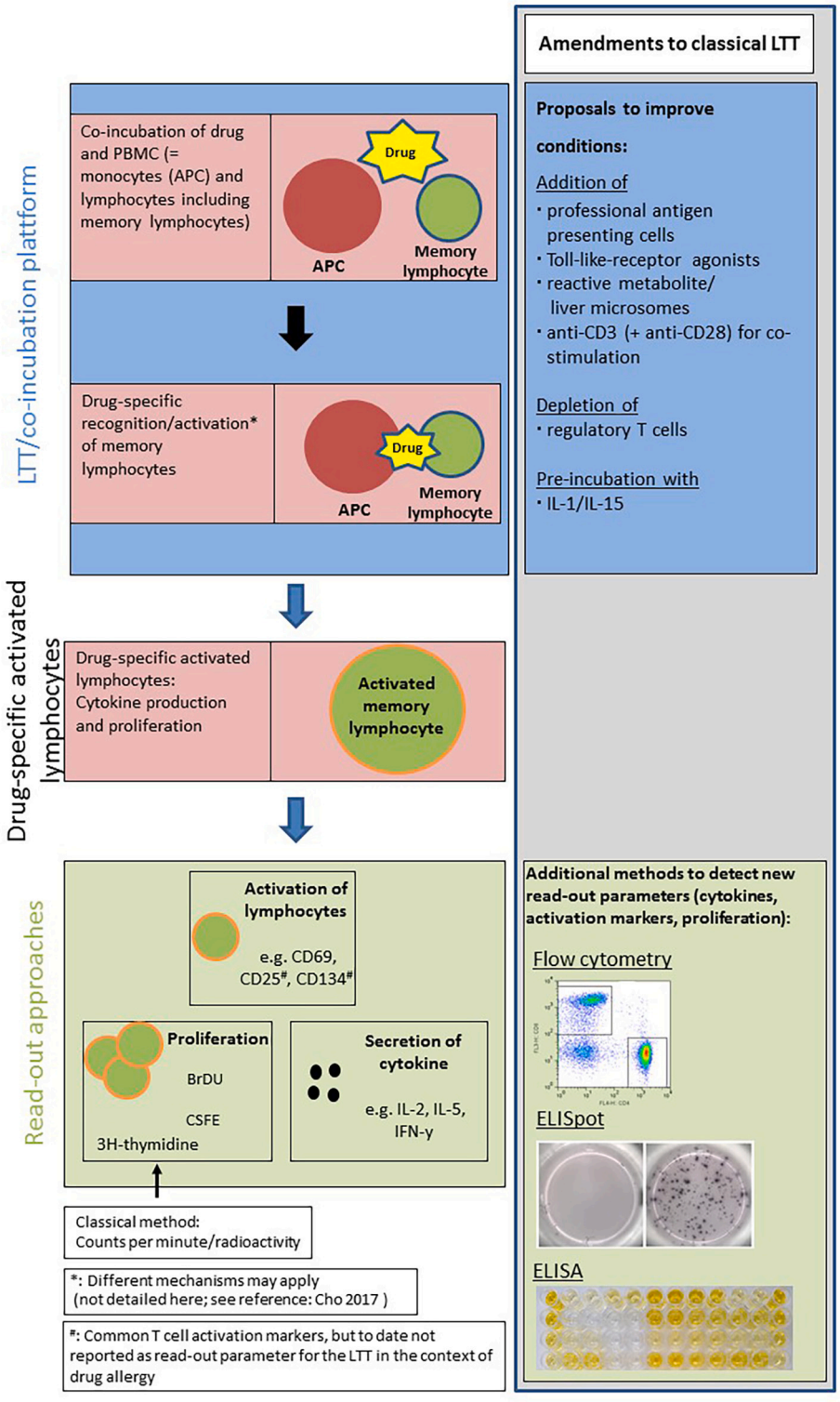

Fig. 2. Methodology of the classical LTT and published amendments. On the left the three different phases of the LTT, i.e., i) the co-incubation of PBMC with the suspected drug (LTT-platform) resulting in ii) drug-specific activation of lymphocytes, and iii) the read-out approaches are shown. On the right amendments to the classical LTT on various levels of the phases are shown. Abbreviations: APC (antigen presenting cell), PBMC (peripheral blood mononuclear cells). * different mechanisms of T cell activation may apply which are not detailed here. Likewise no T cell receptor or MHC-groove is depicted and the interaction is presented in a simplified manner.

advantage is that the LTT is able to detect drug allergic reactions with different immune-mediated pathomechanisms (type I to IV reactions) and to investigate a (limited) number of drugs simultaneously. Moreover, for certain investigated drugs the LTT was reported to be more sensitive for the diagnosis of drug allergies than skin tests (Nyfeler and Pichler, 1997).

The disadvantages include as stated above a varying sensitivity and specificity depending on the clinical phenotype and the drugs. In addition, although the method as such can be standardized, results may vary intra- und inter-individually. Notably, as with other in vitro tests, the detection of an antigen-specific sensitization does not equal an allergy as a sensitization can remain clinically silent. Hence, detection of a sensitization in the LTT should be interpreted in conjunction with the clinical symptoms, the history and if available, other allergy tests (Brockow et al., 2015).

Disclaimer

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Declaration of Competing Interest

The authors declare no conflict of interest.

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